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Identification of amino acid residues important for the Arsenic resistance function of Arabidopsis ABCC1

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Abstract

The Arabidopsis ATP-Binding Cassette (ABC) transporter ABCC1 sequesters arsenic (As)-phytochelatin conjugates into the vacuole, thereby conferring As resistance. Here, we report the results of a screen for phosphorylation-dependent regulation sites of AtABCC1. AtABCC1 variants harboring mutations that replaced amino acid residues Tyr^{682}, Tyr^{709}, Tyr^{822}, Ser^{846}, Ser^{1278}, or Thr^{1408} with alanine conferred reduced resistance and decreased the intracellular As content relative to wild-type AtABCC1 when heterologously expressed in the SM7 yeast strain. This suggested that these mutations compromised the vacuolar sequestration of As by AtABCC1. Furthermore, we found, through a phosphomimic mutant study, that phosphorylation of Ser^{846} is required for the As resistance function of AtABCC1. Our analysis provides a first clue as to the phosphorylation-mediated regulation of AtABCC1 activity.

Keywords: ABC transporter, arsenic, linker phosphorylation.

Introduction

Arsenic (As) is one of the most toxic metal(loid)s in the environment. A natural component of the earth's crust, As is widely distributed throughout the environment, including the air, water, and soil. Furthermore, As is widely used in industry, agriculture, and medicine [1], and is often a cause of industrial pollution. Natural and industrial As pose a serious threat to human health and ecosystems. In South and Southeast Asian countries, such as China, Thailand, and Bangladesh, As-contaminated water and agricultural products cause serious problems for human health [2]. Crops, especially rice, grown in contaminated soils and irrigated with contaminated water, accumulate high levels of arsenic. Consumption of such contaminated agricultural products causes As to accumulate in the human body and results in genotoxic effects, including skin lesions, developmental defects, cardiovascular disease, neurotoxicity, diabetes, and cancer [3].

To reduce the intake of nutritional As, it is necessary to decipher the molecular and physiological mechanisms underlying As accumulation and detoxification in plants. In *Arabidopsis thaliana*, As(V) enters plant root cells via high-affinity phosphate transporters (PTs) [4]. Plant cells reduce As(V) to As(III) using the arsenic reductase HAC1/ATQ1 [5,6], and synthesize phytochelatin (PC) upon exposure to As [7,8]. As(III) is complexed with PC and transported into vacuoles by the ABCC1 and ABCC2 transporters, which belong to the ATP-Binding Cassette transporter family [9,10]. The two ABC transporters are expressed broadly in many plant tissues, and are key mediators of vacuolar sequestration of As in Arabidopsis [10]. In rice, OsABCC1 has been shown to sequester As into the vacuoles of phloem cells in the node, thereby reducing As flow into seeds [11].

AtABCC1 and AtABCC2 have been reported to be involved in the transport of not only As, but also Cd, Hg, and other glutathione (GSH)-conjugated compounds, such as N-ethylmaleimide-GS (NEM-GS), S-(2,4-dinitrophenyl)-GS (DNP-GS), glutathionylated chloroacetamide herbicides, (metolachlor-GS), folates, and antifolates (methotrexate MTX) [12-15]. It is not known how these transporters transport such diverse substrates or which mechanisms regulate their activities. Only a few studies have examined the mechanism underlying the regulation of plant ABC transporters. In animal and yeast cells, ABC transporters have been reported to be regulated at the post-translational level, especially by phosphorylation. Human ABCC7/CFTR is phosphorylated at many amino acid residues, and the phosphorylation is required for its chloride channel function [16]. In budding yeast, the phosphorylation of three different residues of YCF1, a vacuolar Cd-sequestering ABC transporter, has been suggested to positively or negatively regulate its activity [17,18]. The S908A mutated form of ScYCF1 exhibits only 30% of Cd resistance compared to wild-type ScYCF1 [17]. In Arabidopsis, two members of B sub-family ABC transporters, ABCB1 and ABCB19, are also phosphorylated [19,20]. The phosphorylation at the S634 site of ABCB1 regulates its auxin transport activity [20].

We suspected that AtABCC1 might also be regulated by phosphorylation based on three observations. First, it belongs to the ABCC sub-family of ABC proteins, and two

of the ABCC sub-family members, HsABCC7 and ScYCF1, are known to be regulated by phosphorylation [16,17]. Second, AtABCC1 transports metals conjugated with thiols into vacuoles, as does ScYCF1, which is regulated by phosphorylation. Third, two phosphorylation sites in AtABCC1 have already been identified in phosphoproteomics studies; T490 was found to be phosphorylated in response to abscisic acid signaling [21], and T1485 in response to DNA damage [22].

In this study, we describe the results of a screen for amino acid residues that must be phosphorylated to allow AtABCC1 to exert its full function in As detoxification. Among the 29 amino acid residues we chose to mutate, six seemed to be important for AtABCC1-dependent As resistance (Figure 1). Moreover, we showed that Ser^{846}, located in the linker region between nucleotide binding domain 1 (NBD1) and transmembrane domain 2 (TMD2), has to be phosphorylated to confer As resistance. This finding is in agreement with previous studies that showed phosphorylation of the linker region of multiple ABC proteins and its role in regulating their activities [16,17,20]. Our results indicate that phosphorylation of a specific amino acid residue is required for the As resistance activity of Arabidopsis ABCC1. Furthermore, our study suggests that phosphorylation in the linker region is a common mechanism regulating ABCC-type transporters.

Results

Screen for potential phosphorylation sites of AtABCC1

To identify amino acid residues of AtABCC1 that might be phosphorylated, we performed a screen using the SM7 line of yeast. This yeast line lacks YCF1, which is required for yeast arsenic tolerance, but expresses *Triticum aestivum* (wheat) phytochelatin synthase (PCS) TaPCS1, and is hyper-sensitive to As. SM7 has been used to reveal the As-resistance functions of AtABCC1, AtABCC2, and OsABCC1

[10,11]. Thus, this yeast line is ideal for screening many AtABCC1 mutants for their capacity to confer As resistance.

As the linker regions of ScYCF1 and HsABCC7/CFTR have been reported to be important for phosphorylation-mediated regulation [17,20,23,24], we attempted to identify putative phosphorylation sites in this region. However, alignment of the amino acid sequences of the linker regions of AtABCC1, ScYCF1, and HsABCC7/CFTR (Figure 2a) revealed that the amino acid sequences of this region are highly diverse in these transporters, and there were no clearly conserved potential phosphorylation sites. Thus, we evaluated the contribution of all of the serine (Ser^{828}, Ser^{846}, Ser^{858}, Ser^{880}, Ser^{885}, Ser^{899}), threonine (Thr^{815}, Thr^{821}, Thr^{857}, Thr^{877}), and tyrosine (Tyr^{845}) residues in the linker region of AtABCC1 to the As-resistance functions of AtABCC1. We mutated these sites to alanine using site-directed mutagenesis, and expressed the mutated AtABCC1 proteins in the yeast SM7 strain [10].

Among the lines of yeast expressing these mutants, only the one expressing the S846A-mutated form of *AtABCC1* exhibited clearly reduced As resistance when exposed to 100 μM As(III) (Figure 2b), while the other lines exhibited As resistance levels comparable to that expressing native *AtABCC1*. This result suggests that S846 might be important for the As resistance function of AtABCC1.

To test whether the altered activity of the AtABCC1-S846A mutant is indeed due to the defect in phosphorylation of this site, we performed a phosphomimic study in which the Ser^{846} site was mutated to glutamate to mimic its phosphorylated status. We found that As resistance was enhanced in the yeast line expressing S846E-mutated *AtABCC1* to the same extent as in yeast expressing wild-type *AtABCC1* (Figure 2c). The difference in growth of the lines expressing S846E- and S846A-mutated forms of *AtABCC1* was confirmed in liquid culture (Figure 2d). Under control conditions, there was no significant difference between the lines (top panel of Figure 2d); however, when the yeast lines were cultured in medium containing 50 μM As(III) or 100 μM As(III) for 12 hours or longer, the growth of lines harboring empty vector or ABCC1-S846A was significantly lower than that of lines expressing native ABCC1 or ABCC1-S846E. By contrast, there was no significant difference between the growth of native ABCC1 and ABCC1-S846E expressing yeast lines, which indicates that As resistance was restored

in lines expressing the phosphomimic form ABCC1-S846E to similar levels as in lines expressing native ABCC1. It should be noted that a similar amount of protein existed in yeast cells expressing *AtABCC1-S846E, AtABCC1-S846A,* and native *AtABCC1*, as shown in the Western blot using V5 tag antibody (Figure 2e). These results indicate that phosphorylation at the S846 site is necessary for full function of AtABCC1 as an As resistance factor in the SM7 yeast.

To identify more potential phosphorylated residues, we performed a prediction analysis of the putative phosphorylation sites of AtABCC1 using the online prediction software PhosPhAt (http://phosphat.uni-hohenheim.de/). According to the prediction, 56 sites might be phosphorylated (Figure 3a). Among these, we selected 13 sites (Tyr^{682}, Tyr^{709}, Ser^{741}, Ser^{749}, Tyr^{822}, Thr^{895}, Tyr^{906}, Ser^{1084}, Ser^{1088}, Ser^{1278}, Thr^{1408}, Thr^{1434}, and Thr^{1454}) that are conserved among the AtABCC1 homologs in multiple organisms (Figure 3b), since we speculated that the conserved residues most likely correspond to conserved regulatory domains. In addition, we included two phosphorylated sites (Thr^{490} and Thr^{1485}) previously identified in LC/MS/MS experiments [21,22] (Figure 3a). The 15 selected residues were mutated to alanine using site-directed mutagenesis, and the mutated genes were introduced into the SM7 yeast line. As shown in Figure 4, yeast expressing any of five alanine-mutated forms of AtABCC1 (Y682A, Y709A, Y822A, S1278A, or T1408A), but not the remaining alanine-mutated forms, showed strongly reduced As resistance compared to yeast expressing native AtABCC1 when exposed to 100 µM As(III).

To test whether the altered activities of the AtABCC1 mutants were indeed due to the phosphorylation of these sites, we performed a phosphomimic study. The tyrosine sites were mutated to phenylalanine to mimic their non-phosphorylated form (Y682F, Y709F, and Y822F). When the tyrosine sites were mutated to phenylalanine, yeast cells expressing these forms grew as well as yeast expressing wild-type AtABCC1 (Figure 4b). This result indicates that phosphorylation of these three tyrosine sites is not a prerequisite for conferring As tolerance. However, we cannot exclude the possibility that phosphorylation of these sites might be necessary to inactivate the As tolerance function of AtABCC1. We could not test this possibility further, since there is no suitable amino acid to mimic phosphorylated tyrosine. The T1408 and S1278 sites were mutated

to glutamate (S1278E and T1408E) to mimic their phosphorylation status. We found that S1278E and T1408E substitutions did not recover As resistance (Figure 4b). This result indicates that the loss of As resistance by the mutations of these sites was not due to a defect in phosphorylation.

Recently, the three serines (Ser^{1233}, Ser^{1234} and Ser^{1236}) of the 'SSGS' motif of AtABCC1 were reported to be phosphorylated by CKII, based on the findings of an *in vitro* experiment [25]. Therefore, we mutated these three serine residues in AtABCC1 to alanine and expressed this mutant in SM yeast. However, we could not detect any difference in As resistance of yeast lines expressing the mutant and wild-type forms of AtABCC1 (Figure 4c).

The effect of mutations of AtABCC1 on As concentration

To test whether mutations of AtABCC1 affect As concentrations in the transgenic SM7 yeast cells, we analyzed the As concentration using ICP-MS. We found that the yeast cells expressing S846E AtABCC1 mutant contained similar As levels as the cells harboring wild-type AtABCC1, whereas the cells harboring S846A-mutated AtABCC1 exhibited a lower As concentration (Figure 5a). Yeast cells harboring a mutation that replaced any of the six sites shown to be important for the As resistance function of AtABCC1 with alanine (Y682A, Y709A, Y822A, S846A, S1278A, T1408A) also exhibited significantly lower As concentrations than did the cells expressing wild-type AtABCC1 (Figure 5b, $p<0.01$). This result is in good agreement with our yeast growth results (Figures 2 & 4), and further supports the possibility that S846 is an important site regulating the activity of AtABCC1 via phosphorylation.

Discussion

Many plant ABC transporters have been characterized, and their physiological functions revealed [26]. However, the regulatory mechanisms of plant ABC transporters are poorly understood. Based on several lines of experimental evidence, we report here that the phosphorylation of Ser^{846} is required for the AtABCC1 activity. First, yeast cells

198 expressing the mutant protein AtABCC1-S846A exhibited reduced As resistance
199 compared to cells expressing wild-type AtABCC1 (Figure 2). Second, the phosphomimic
200 mutation AtABCC1-S846E recovered the As tolerance level of the transgenic yeast to
201 that of yeast expressing wild-type AtABCC1 (Figure 2c & d). Third, the As concentration
202 of yeast expressing AtABCC1-S846A was as low as that of yeast containing the empty
203 vector, whereas the As concentration of yeast expressing AtABCC1-S846E was the
204 same as that expressing wild-type ABCC1 (Figure 5a).

205 Ser^{846} is located in the linker region between the NBD1 and TMD2 of ABCC1.
206 Phosphorylation of this region has been reported to play an important role in regulating
207 ABCB and ABCC transporters [27]. In mammalian ABCC7s/CFTRs, the linker region,
208 also called the R domain, is highly phosphorylated by different protein kinases [16,27].
209 Mutation of the phosphorylation sites of ABCC7 at the linker region or of the kinase that
210 phosphorylates these sites causes serious diseases in humans [28]. Linker region
211 phosphorylation also seems to play important roles in the proper function of ABCB
212 transporters [27]. *Arabidopsis* ABCB1 is phosphorylated at the Ser^{634} site in the linker
213 region, and mutation of S634 to alanine reduced IAA export to 50% of wild-type levels,
214 while the phosphomimic mutation S634E increased it to 150%, when expressed in yeast
215 cells [20]. Yeast YCF1 is another example of ABC transporter proteins with the linker
216 region that is phosphorylated; phosphorylation of the linker residues Ser^{908} and Thr^{911}
217 positively regulates the function of YCF1 [17]. Thus, phosphorylation is a common
218 regulatory mechanism of ABC transporters in different organisms. This is rather
219 surprising since the amino acid sequences of the linker regions of ABC proteins
220 regulated by phosphorylation are not conserved (Figure 2a). The S846 site is not highly
221 conserved among ABCC1 homologs of many organisms, either; among AtABCC1
222 homologs in *Arabddopsis thaliana, Brassica rapa*, *Oryza sativa*, *Zea mays, Hordeum*
223 *vulgare,* and *Schizosaccharomyces pombe*, only *AtABCC2* and the *Brassica rapa*
224 *ABCC1* homolog have a serine at this site (Supplementary figure 1). Interestingly, the
225 other *ABCC1* homologs have a conserved serine residue at the position corresponding
226 to E853 of *AtABCC1*. It is tempting to speculate that this serine residue close to the
227 S846 site might be phosphorylated to regulate the activities of *ABCC1* homologs, and

228 that the linker regions regulated by phosphorylation share structural similarities among
229 different ABC transporters. However, these possibilites remain to be evaluated by
230 further studies.

231 Among the 29 amino acid sites we chose to mutate in this current study, only 6
232 affected As resistance when mutated to alanine (Figures 2 & 4). This is surprising, since
233 our choice was based on the alignment of ABCC1 orthologues of different organisms,
234 phospho-proteomics data, and previous experimental results on phosphorylation of
235 similar ABC proteins. Furthermore, among these residues, only one was found to be
236 putatively phosphorylated based on our phosphomimic results and involved in As
237 resistance (Figure 2c). By contrast, 3 phosphorylation sites were detected in ScYCF1
238 [17,18] and 10 in HsABCC7, also known as CFTR [16]. However, the absence of
239 altered phenotypes by single amino acid mutation does not exclude the possibility that
240 these residues are involved in phosphorylation-mediated regulation. Combinations of
241 multiple mutations might be needed to reveal such a phenotype. It remains to be
242 elucidated whether there is only one phosphorylation site that affects AtABCC1 activity,
243 whether there are additional phosphorylation sites alone or in combination that
244 modulate the activity of the transporter, or whether different sites need to be
245 phosphorylated to transport different substrates.

246 In mammalian cells, ABC transporters are mostly phosphorylated by PKA, PKC,
247 and CKII kinases [29]. Arabidopsis ABCB1 and ABCB19 transporters have been
248 reported to be phosphorylated by AGC kinases, which are the plant orthologues of
249 mammalian protein kinase groups A, G, and C. Recently, the 'SSGS' motif of AtABCC1
250 was shown to be phosphorylated by CKII *in vitro* [25]. However, no *in vivo* evidence was
251 provided for the actual phosphorylation of the motif, nor was there a functional study to
252 evaluate the importance of this phosphorylation. We mutated the three serine residues
253 in the 'SSGS' motif, but did not observe any difference in the As resistance function of
254 the resulting ABCC1 proteins when expressed in SM7 yeast (Figure 4c), which indicates
255 that the phosphorylation of this motif is not important for conferring AtABCC1-dependent
256 As tolerance. Furthermore, our results do not support major roles of the phosphorylation
257 of T490 or T1485 in As resistance of AtABCC1, since we did not detect any change in

the As resistance of the corresponding mutants (Figure 4a). However, our results do not exclude the possibility that these sites are phosphorylated in response to ABA or DNA damage, and have a role in the response to these stimuli, because they were identified as being phosphorylated in comparative phosphoproteomics studies of an ABA-signaling mutant or DNA damage response mutant and the wild type [22 and 23].

In the present study, we identified Ser^{846} as a putative phosphorylation site of AtABCC1. Ser^{846} and its neighboring residues Glu^{847} and Glu^{848} form an 'SEE' motif, which is a putative phosphorylation motif of CKII kinase [30]. It would be interesting to test whether Ser^{846} is indeed phosphorylated by a CKII-like kinase *in planta*. In addition to the putative phosphorylation site at Ser^{846}, we also identified 5 additional sites that are important for the As resistance of AtABCC1, namely, Tyr^{682}, Tyr^{709}, Tyr^{822}, Ser^{1278}, and Thr^{1408}. When these sites were mutated to alanine, AtABCC1-mediated As resistance was significantly reduced (Figure 4a). The loss of As resistance caused by mutation of Tyr^{682}, Ser^{1278}, or Thr^{1408} might be due to the disruption of the ATP binding and hydrolysis activity, because Tyr^{682} is located next to the Q-loop of NBD1, Ser^{1278} is located in the Walker A motif of NBD2, and Thr^{1408} is located near the D-loop of NBD2. These motifs have been shown to be necessary for ATP binding and ATP hydrolysis-driven conformational changes [31]. However, the Tyr^{709} and Tyr^{822} sites are located at a distance from any of the known ABC protein motifs present in AtABCC1. The reason for the reduced As resistance of these ABCC1 mutants is unclear. Further investigations are needed to decipher the functions of these amino acid residues of AtABCC1 in As resistance, and to test the possibility that they might be important for maintaining the physical and chemical properties of AtABCC1, including polarity and three-dimensional structure.

In conclusion, using yeast to screen for AtABCC1 mutants, we identified six sites important for the As resistance function of AtABCC1. Among these sites, phosphorylation of Ser^{846}, located at the linker region of AtABCC1, was required for AtABCC1-mediated As resistance. Thus, similarly to other ABCC proteins in other organisms, Arabidopsis ABCC1 may also be regulated by phosphorylation in the linker region. However, mutations in other potential sites in the linker region did not change

the activity of AtABCC1 with respect to As detoxification ability, suggesting that either the number of amino acid residues in the region regulating the protein activity by phosphorylation may not be as many as in other ABCC proteins or that AtABCC1 is regulated in a substrate-specific manner. These differences are intriguing, and suggest that different mechanisms regulate the activity of plant ABCC transporters. Thus our study lays a basis for further studies of plant-specific regulatory mechanisms of ABC transporters.

Materials and methods

Site-directed mutagenesis

Site-directed mutagenesis was performed by overlapping PCR. Primers used are listed in Supplementary Table 1. The pNEV vector containing *AtABCC1* cDNA (Song et al., 2010) was used as the template. The PCR products were digested with DpnI to eliminate the template plasmid and then introduced into DH5α competent cells. The mutations were confirmed by sequencing.

Yeast growth assays

The yeast SM7 strain expressing the *TaPCS* gene [8] was used to evaluate the As tolerance imparted by various mutant forms of AtABCC1. Plasmids were introduced into the yeast cells by the LiAc/PEG/ssDNA method [32]. Yeast complementation was tested using drop spotting assays. Yeast cultures were diluted to OD_{600}=0.1 and sequentially diluted to 0.01, 0.001, and 0.0001 and spotted onto SD-URA media with or without 100 μM As(III). To compare the growth rates of different yeast lines, yeast cells were inoculated into 2 ml SD-URA medium and grown at 30ºC on a shaker overnight. Then the cells were diluted to OD_{600}=0.1 in 10 ml fresh SD-URA medium supplemented with or without As(III) and grown again at 30ºC on a shaker. Growth rate was monitored by measuring OD_{600} values of the cultures at 2, 4, 6, 9, 12, and 24 hours.

As content measurement

To measure the As content in yeast, yeast colonies were grown in liquid SD-URA media at 30ºC for overnight. Then, yeast cultures were transferred into fresh 10 ml SD-URA media containing 100 µM As(III) to a cell density of OD_{600}=1. Cells were grown for 7 hours, collected by centrifugation at 3000 rpm for 2 min, and washed three times with ice-cold Milli-Q water. Then the yeast cells were resuspended in 550 µL Milli-Q water and OD_{600} was determined. At 100 µM As(III), the final cell density did not differ much between different samples. Subsequently, 500 µL of the cells were taken, dried, and digested using 1 mL nitric acid at 140ºC for 10 hours. The As concentrations of the samples were measured using inductively coupled plasma mass spectrometry (ICP-MS).

Immunoblot analysis

Native and mutant *AtABCC1* genes were cloned into pYES2/NTC vector as C-terminal fusions with the V5 tag. The vectors were introduced into the yeast SM15 line [33], which is a line selected from the SM7 line of yeast, for active growth in galactose-containing medium. Yeast lines harboring the various *AtABCC1* constructs were grown in SG-URA medium at 30ºC for overnight, and their proteins were extracted as described by Song et al. [34]. The total protein preparation was centrifuged at 10,000g for 10 min at 4ºC, and then the supernatant was centrifuged at 100,000g for 1 h at 4ºC to obtain the membrane proteins. Twenty microgram of membrane proteins was separated by SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked with blocking buffer that contained 7.5% non-fat milk in 1×TBST (0.1% (v/v) Tween 20 in 1× Tris buffered saline) for 1 h at room temperature, washed for 5 min with 1×TBST, and incubated with anti-V5 antibody overnight at 4ºC. The membrane was then washed three times with 1×TBST at room temperature and incubated with anti-mouse secondary antibody for 1 h at room temperature. The protein band was detected by chemiluminescence using ECL reagent.

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Contribution

JZ, JUH, WYS, and YL conceived the study; JZ performed experiments and analyzed data; JZ, EM, and YL wrote the manuscript.

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Figure legends

Figure 1. Map showing the positions of amino acid residues of AtABCC1 tested in this paper for potential phosphorylation-dependent regulation of AtABCC1 function in As resistance.

Red lines indicate the nucleotide-binding domains (NBDs). Blue bars represent transmembrane domains (TMDs). The green line indicates the linker region between NBD1 and TMD2. Dots represent the amino acid residues we tested in this paper. Blue dots represent the amino acid residues we identified as being important for As resistance and accumulation. Mutations of other residues to alanine did not change the As resistance function of AtABCC1 when expressed in the SM7 line of yeast.

Figure 2. Phosphorylation of AtABCC1 at S846 is necessary for arsenic resistance.

(a) Alignment of predicted linker regions of AtABCC1, ScYCF1, and HsABCC7. The linker region of AtABCC1 (amino acid residues 815-915) was predicted using the PhosPhAt database, and the linker region of ScYCF1 (853-951) and HsABCC7 (647-858) were obtained from the Uniprot database. The amino acid residues with a gray background represent similar amino acids and those with a black background represent identical amino acids. The serine residue in the red circle represents the phosphorylation site identified in the present study. Green circles represent the serine, threonine, and tyrosine residues in the linker region of AtABCC1 that we tested. Blue circles represent the phosphorylated residues with functional importance identified in ScYCF1 and HsABCC7/CFTR. (b) A screen for putative phosphorylation sites (amino

acid residues highlighted in green and red circles in (a)) in the linker region of AtABCC1. Yeast cells grown in liquid culture were spotted on SD-URA (pH 7) in the absence or presence of 100 µM As(III), and grown for 2 days. (c) Phosphomimic analysis of Ser^{846}, which was mutated to alanine or glutamic acid to mimic its non-phosphorylation or phosphorylation status, respectively. (d) Growth curves of yeast strains (harboring empty vector, native AtABCC1, AtABCC1-S846A, or AtABCC1-S846E) cultured in SD-URA medium supplemented without (control, top panel) or with 50 µM As(III) (middle panel) or 100 µM As(III) (bottom panel). OD_{600} values shown are mean ± SD of three biological replicates. Significant differences among different yeast lines at the time point of 24 hours are indicated as different letters (*P<0.01*, ANOVA, Turkey test). (e) Immunoblot showing protein levels of native and Ser^{846}-mutated AtABCC1 in SM15 yeast. For each strain, 20 µg of microsomal proteins was loaded. Anti-V5 antibody was used to detect the AtABCC1 protein fused with V5 tag.

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(a) As concentrations in yeast transformed with native AtABCC1, its non-phosphorylation mutant S846A, or phosphomimic mutant S846E. The same yeast lines as used in Figure 1(c) were used for this analysis. (b) As concentrations in yeast transformed with native AtABCC1 or its non-phosphorylated mutants reduced in As tolerance, as shown in Figure 3. Yeast cells were grown in liquid SD-URA medium overnight and diluted to OD_{600}=1 in 10 ml fresh SD-URA medium with or without 100 µM As(III). After 7 h treatment, the yeast cells were washed three times with ice-cold sterilized distilled water. The yeast cells were digested using nitric acid and their As content was measured using ICP-MS. The As concentration was normalized based on the OD_{600} value. Values are mean ± SD of six biological replicates. ** $P < 0.01$ (Student's *t*-test) relative to the corresponding empty vector (EV) control value.

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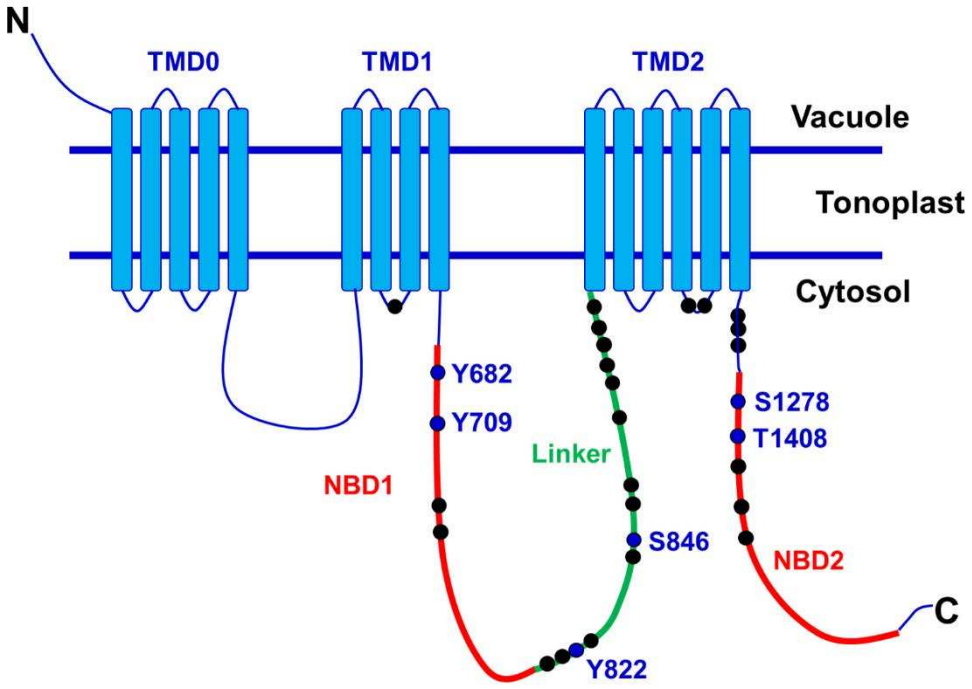

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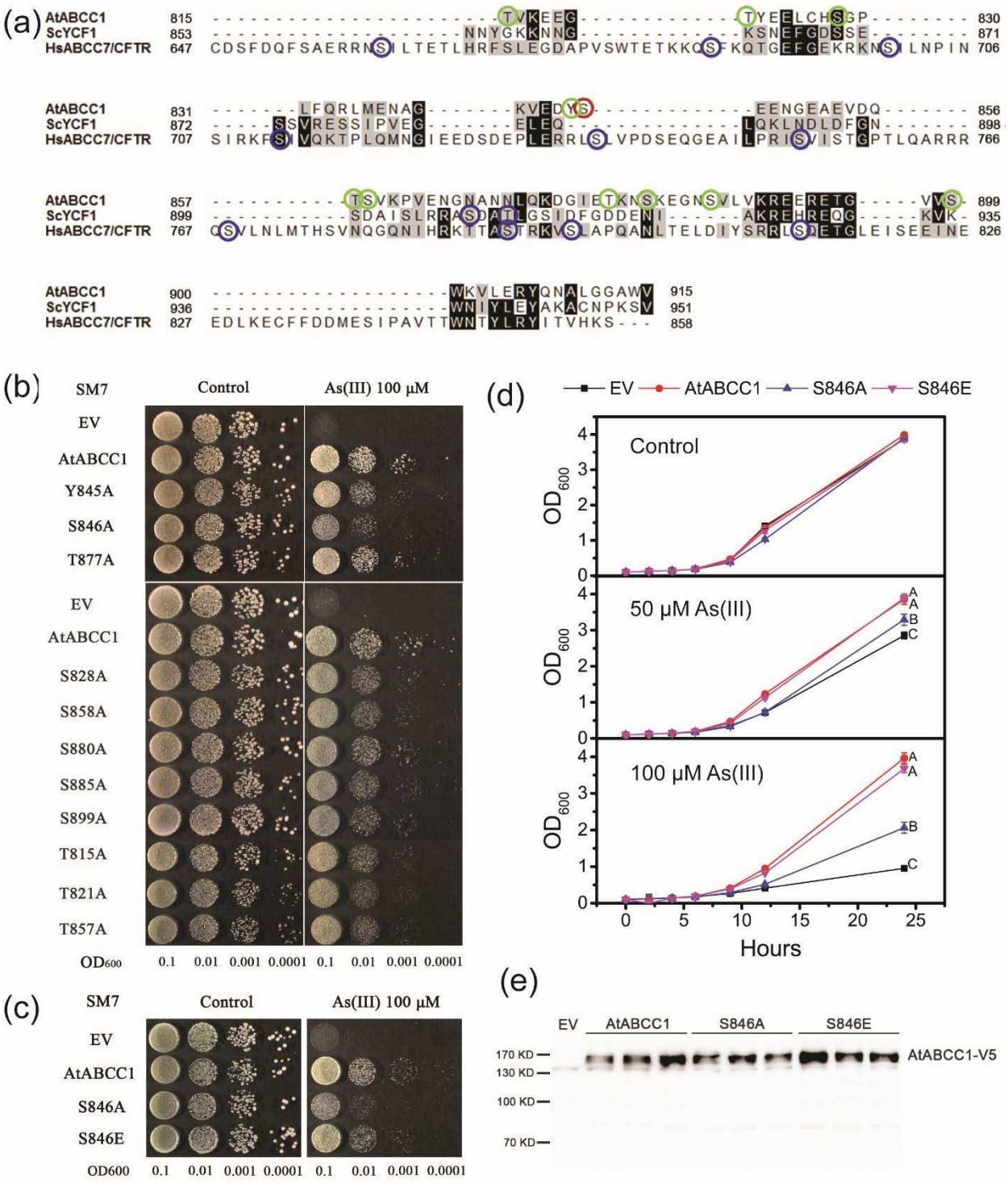
(a)
AtABCC1
ScYCF1
HsABCC7/CFTR
(b)
SM7
Control
As(III) 100 μM
EV
AtABCC1
Y845A
S846A
T877A
S828A
S858A
S880A
S885A
S899A
T815A
T821A
T857A
OD600
0.1 0.01 0.001 0.0001
(c)
OD600
(d)
EV
AtABCC1
S846A
S846E
Control
50 μM As(III)
100 μM As(III)
OD600
Hours
(e)
170 KD
130 KD
100 KD
70 KD
AtABCC1-V5

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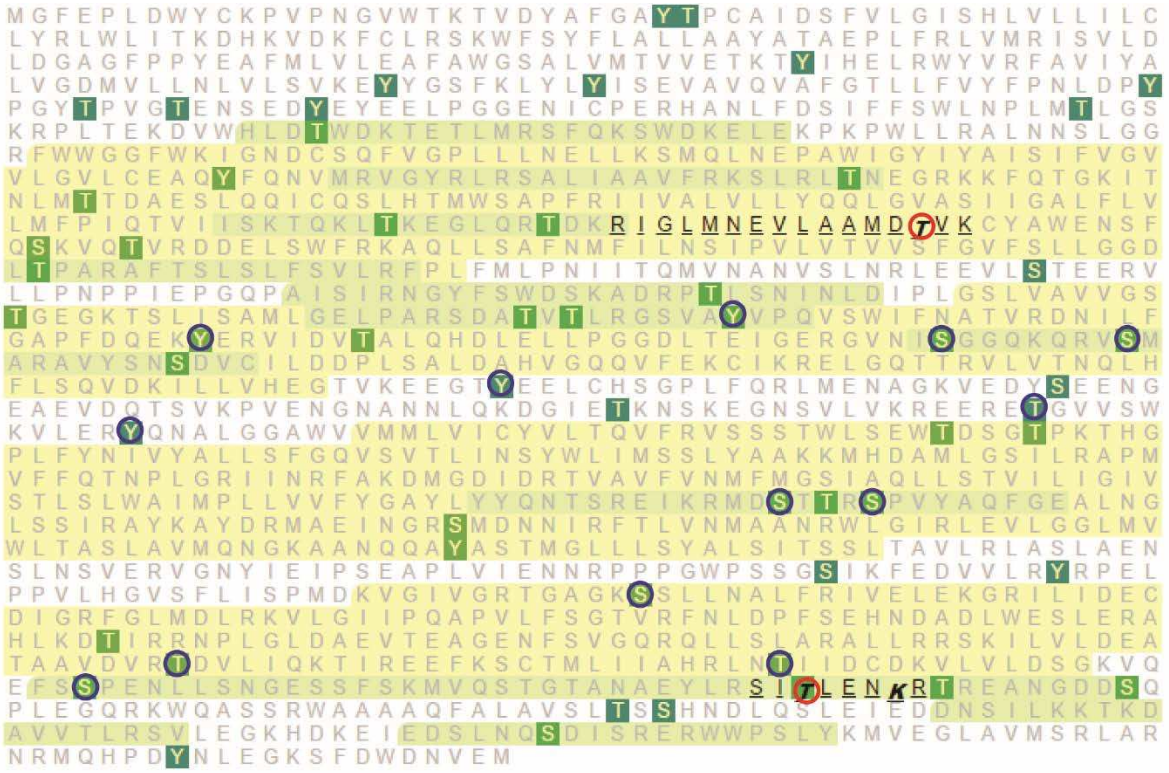
(a)



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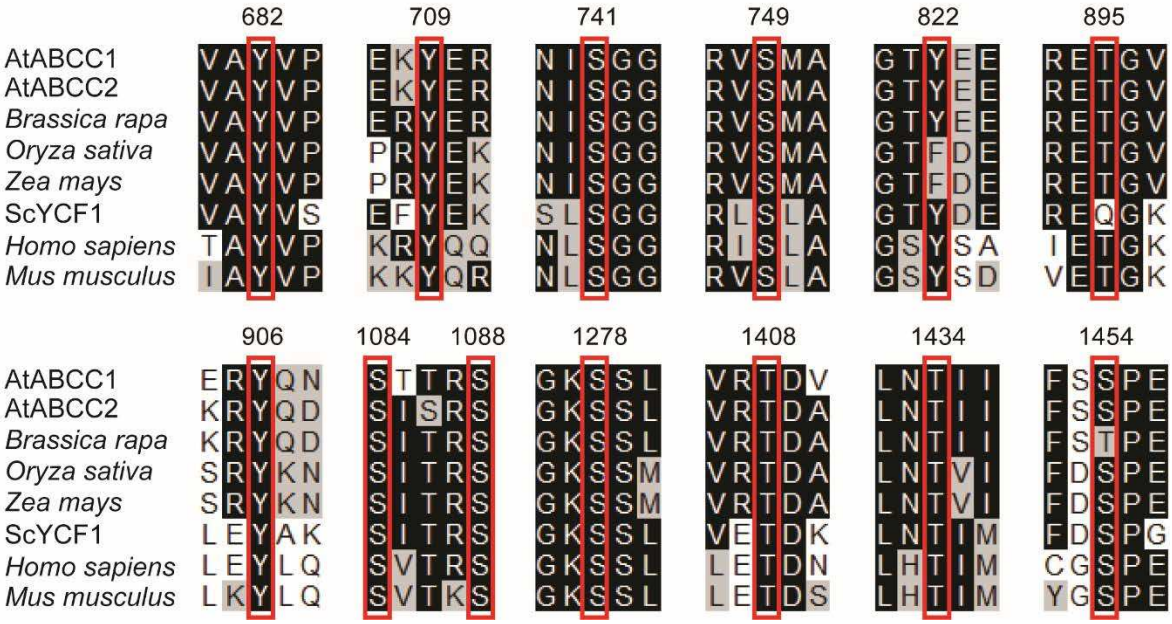
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AtABCC1
AtABCC2
Brassica rapa
Oryza sativa
Zea mays
ScYCF1
Homo sapiens
Mus musculus
906
1084
1088
1278
1408
1434
1454

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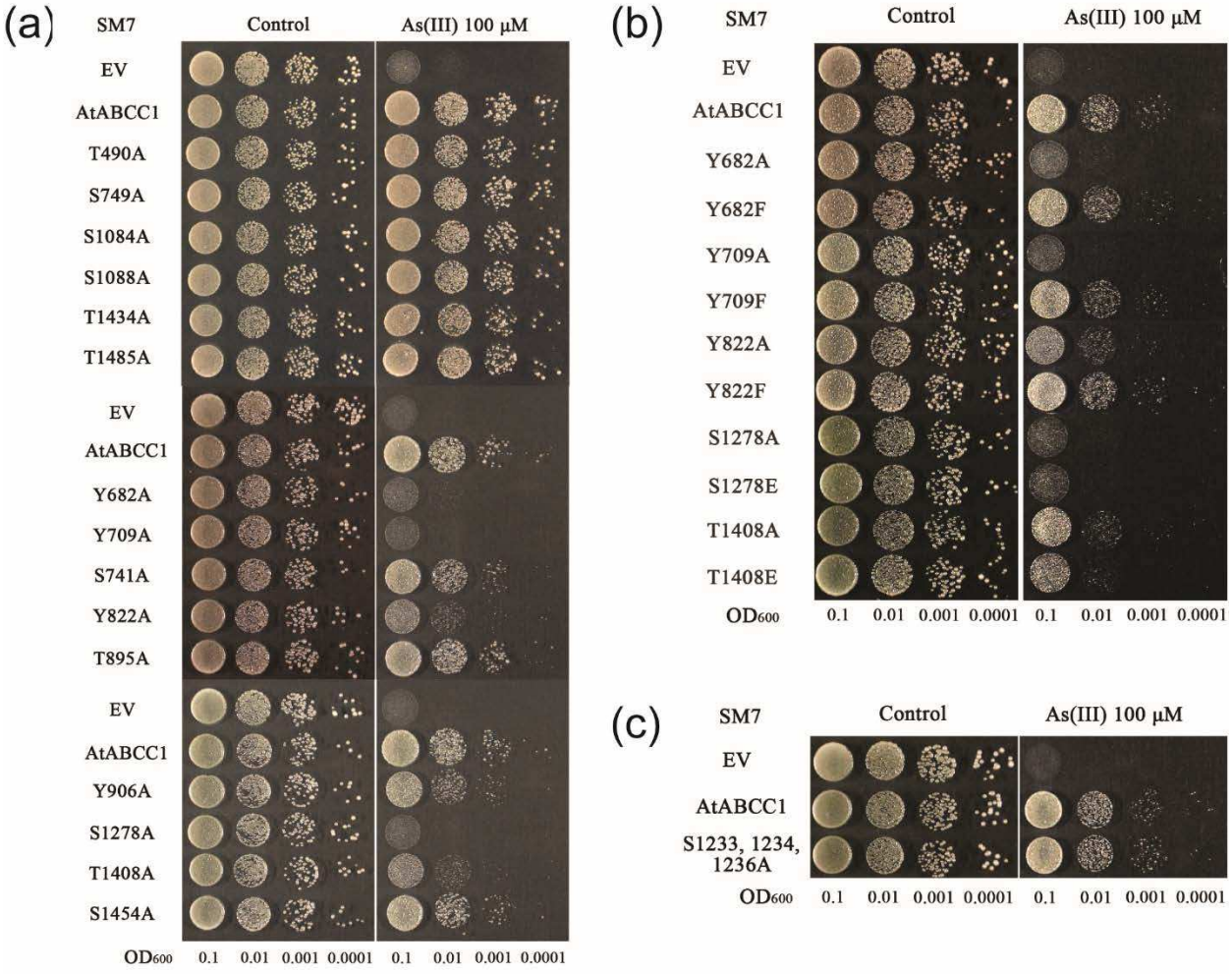

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